

## Tetanus Toxin and Botulinum A Toxin Inhibit Release and Uptake of Various Transmitters, as Studied with Particulate Preparations from Rat Brain and Spinal Cord\*

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**Summary.** The effects of tetanus toxin and botulinum A toxin on the uptake and evoked release of various neurotransmitters were studied using particles from rat forebrain, corpus striatum and spinal cord.

**1. Uptake.** Tetanus toxin partially inhibits the uptake of glycine and choline into spinal cord synaptosomes. The effect on glycine uptake becomes statistically significant after a lag period of 60–120 min. It is no longer present when the toxin is heated, antitoxin-treated or toxoided. The inhibition by botulinum A toxin of choline uptake into spinal cord synaptosomes is weak but measurable, that of glycine uptake is at the borderline of detection.

The uptake of GABA into forebrain cortex synaptosomes is slightly inhibited by tetanus toxin but hardly by botulinum A toxin. The effects of tetanus toxin and botulinum A toxin on the uptake of noradrenaline into striatal synaptosomes are negligible.

**2. Release.** Tetanus toxin inhibits the potassium (25 mM) evoked release of radioactivity from rat forebrain cortex particles preloaded with labelled neurotransmitters. The sensitivity decreases in the following order: Glycine > GABA > acetylcholine. The toxin also inhibits the release of radioactivity from striatal particles preloaded with labelled noradrenaline. It is always 10–50 times more potent on spinal cord than on brain particles. The sensitivity of the evoked release from the spinal cord decreases in the order glycine > GABA > acetylcholine > noradrenaline.

The toxin is identical with the causative agent because toxin-antitoxin complexes, toxoid and heated toxin do not influence the release from particles preloaded with glycine (spinal cord), GABA (forebrain) and noradrenaline (striatum).

Botulinum toxin resembles tetanus toxin by its ability to diminish the release of radioactivity from preloaded forebrain (acetylcholine > GABA), striatal (noradrenaline), or spinal cord (glycine) particles. The botulinum toxin effect on the striatum (noradrenaline) and on the spinal cord (glycine) is due to its neurotoxin content.

The identity of the toxin and the causative agent has been established by preheating and preincubation with antitoxin.

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\* The essentials of this communication, which is part of the thesis of I. Heller, have been presented at the Joint Meeting of the Scandinavian and German Pharmacological Societies, Travemünde 1980 (Bigalke et al. 1980)

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It is proposed that a) tetanus and, however to a much lesser degree, botulinum A toxin act in a basically similar manner on a process underlying the function of synapses in general, and b) the pronounced sensitivity of glycine and GABA release from spinal cord, together with the axonal ascent of tetanus toxin, may be crucial in the pathogenesis of tetanus.

**Key words:** Tetanus toxin — Botulinum A toxin — Neurotransmitter — Uptake — Release

### Introduction

Both tetanus toxin and, to a lesser degree, botulinum A toxin inhibit synaptosomal choline uptake (for detailed discussion see Habermann et al. 1981) and depress the potassium evoked release of acetylcholine from brain particles and slices (Bigalke et al. 1981). Botulinum A toxin and, to a lesser degree, tetanus toxin interrupt cholinergic transmission in the isolated mouse diaphragm (Habermann et al. 1980b) and in the ileum strip of the guinea-pig (Bigalke and Habermann 1980) by a presynaptic action.

Compared with the cholinergic system, other transmitter systems are less defined as to their sensitivity to clostridial neurotoxins. According to Rand and Whaler (1965) and Holman and Spitzer (1973) botulinum A toxin paralyzes the noradrenergic innervation of the mouse vas deferens. In our hands, neither tetanus nor botulinum A toxin impeded the noradrenergic transmission in the mouse vas deferens, the rat anococcygeus muscle, or the cat nictitating membrane (Habermann et al. 1980a).

Some evidences exist for interferences, particularly of tetanus toxin, with central transmitter systems. Inhibition of glycine and GABA release has been deduced from electrophysiological experiments in cats (Gushin et al. 1970; Curtis et al. 1973). Osborne and Bradford (1973) prepared synaptosomes from poisoned rats and added tetanus toxin in vitro. Upon electrical stimulation the synaptosomes released smaller amounts of glycine, GABA and glutamate than non-poisoned controls. Collingridge et al. (1980) injected tetanus toxin (1 µl corresponding to about 80 ng) into the substantia nigra. GABA release evoked by high potassium was diminished from slices prepared from the poisoned as compared with the intact side. In a similar set-up, tetanus toxin was found to impair the release of GABA and dopamine, but not of 5-hydroxytryptamine or acetylcholine from striatal tissue poisoned in vivo. As to the in vitro uptake of transmitters, in

vivo poisoned tissue did not differ from samples taken from the contralateral side.

We have now extended our earlier *in vitro* studies, which were restricted to the cholinergic system, to transmitters of various classes, in particular noradrenaline, glycine and GABA.

## Materials and Methods

### Substances

Tetanus toxin ( $LD_{50}$ , mouse, s.c. 3 ng/kg) was from Institut Pasteur, Paris. Botulinum A toxin, "crystalline" ( $LD_{50}$  3–5 ng/kg) was from Dr. Schantz, Food Research Institute, Madison, WI, USA. Neurotoxin was prepared from botulinum toxin according to Moberg and Sugiyama (1978). Its present  $LD_{50}$  was 7.5 ng/kg. Its haemagglutinin content was below the detection limit of 0.4%; as tested by haemagglutination of human blood cells. Tetanus toxoid (347 IU/mg protein), tetanus antitoxin (horse, Fermo antiserum, 5000 U/ml) and botulinum antitoxin (horse, 750 U/ml) were from Behringwerke, Marburg, FRG. Before use they were dialyzed against Krebs-Ringer-Hepes with the composition given below. All toxins were diluted in the presence of 0.1% bovine serum albumin (BSA; Behringwerke, Marburg, FRG). [ $^{14}C$ ]-glycine (107 mCi/mmol), [ $^3H$ ]-choline (84 Ci/mmol), [ $^3H$ ]-noradrenaline (11.8 Ci/mmol), [ $^3H$ ]-5-hydroxytryptamine (14 Ci/mmol), [ $^3H$ ]-gamma-aminobutyric acid (60 Ci/mmol), [ $^3H$ ]-dopamine (43 Ci/mmol) and [ $^{14}C$ ]-serine (174 mCi/mmol) were from Amersham-Buchler, Braunschweig, FRG. All tracers were tested for radiochemical homogeneity by thin layer chromatography. Radioactivity was counted in a Packard liquid scintillation Tri-Carb 2660 counter with automatic external standard.

Synaptosomes ( $P_2$ -fraction) for uptake studies were prepared from rat forebrain cortex, striatum and spinal cord as described previously (Habermann et al. 1981). Briefly, rats (Wistar AF-Han, about 150 g body weight) were killed by bleeding under slight ether anaesthesia, the organs mentioned removed quickly, dissected on ice, homogenized in 10 vol (v/w) 0.32 M sucrose and centrifuged at  $1,000 \times g$  for 10 min. The supernatant was centrifuged at  $11,000 \times g$  for 30 min, the sediment resuspended in sucrose and resedimented. The final pellet was brought cautiously into the original volume of Krebs-Ringer solution buffered (pH 7.4) with Hepes (KR-H). This solution, which was used throughout, contained NaCl 130, KCl 4.75,  $CaCl_2$  2.54,  $KH_2PO_4$  1.19,  $MgSO_4$  1.2, glucose 11, Hepes 10 (all in mM). The gas phase was air.

### Methods

**1. Uptake.** In general, the two-step design used for choline uptake (see Habermann et al. 1981) was followed. In the first step 0.2 ml of the synaptosome dilutions in KR-H were shaken at 37 °C for 2 h (if not otherwise stated) with 0.1 ml toxin in KR-H containing 0.1% BSA. In the second step 0.1 ml labelled transmitter in KR-H was added and its uptake was stopped by dilution with 3.5 ml ice-cold KR-H containing 0.1 mM of the respective unlabelled transmitter, followed by filtration through two layers of Whatman GF-C glass fibre filters. The filters were washed twice with the same volumes, shaken for at least 2 h in the cold with 10 ml Quicksint and counted for radioactivity.

Minor changes of the protocol referred to the washing between the first and the second step which was performed with [ $^3H$ ]-choline only. The optimum concentration of synaptosomes in the first step, and of transmitter, time and temperature required in the second step was determined for each transmitter in preliminary experiments. The temperature and time duration of the experiments were selected so as to give an uptake proportional to time. Protein incubate varied from 0.15 to 0.6 mg, the transmitter concentration from 50 to 2,500 nM, the duration of the second step between 3 and 15 min, and the temperature between 30 °C (glycine) and 37 °C (all others), dependent on the transmitter used.

**2. Release.** Synaptosomes were unsuitable for superfusion in our apparatus because they were not completely retained on the Sephadex layer (see below). Therefore slices ( $300 \times 300 \mu m$ ) were prepared using a

Mellwain tissue chopper, suspended in 20 vol KR-H, triturated by 60 strokes with an Eppendorf (1 ml) pipet and sedimented with KR-H to remove the fines (Bigalke et al. 1981). The particles were suspended in KR-H (1% w/v in the case of GABA and 5% otherwise with respect to tissue weight). To remove easily releasable intrinsic transmitter, aliquots (300  $\mu l$ ) were shaken in polypropylene tubes for 30 min at 37 °C, diluted with 3.5 ml ice cold KR-H and centrifuged shortly at  $4,500 \times g$ . The sediment was resuspended by vortexing for 15 s in 0.3 ml KR-H containing the radioactive transmitter together with, if necessary, unlabelled transmitter to yield a final concentration of 1.0  $\mu M$  GABA, 1.5  $\mu M$  glycine, 0.2  $\mu M$  noradrenaline, or 0.2  $\mu M$  choline. The radioactivity was approximately 1  $\mu Ci/ml$  for the tritiated substances and 150 nCi/ml for [ $^{14}C$ ]-glycine. For stabilization, ascorbic acid (10  $\mu M$ ) and iproniazid (5  $\mu M$ ) were added to the noradrenaline solutions, and aminooxyacetic acid (10  $\mu M$ ) to the GABA solutions, whereas no inhibitor was added in the experiments with [ $^3H$ ]-choline. After 60 min, uptake was terminated by dilution with 3.5 ml icecold KR-H and centrifugation. The particles were vortexed with 300  $\mu l$  KR-H containing 0.1% BSA with or without the toxins and incubated with shaking for a further 2 h. Then the samples were diluted and centrifuged a third time, resuspended in KR-H and transferred into the superfusion chamber which contained a 500  $\mu l$  Sephadex G-25 bed. When prepared from forebrain, the particles remained on the Sephadex layer. With spinal cord particles, the initial filtrate was slightly turbid, indicating some loss of tissue matter.

The loaded Sephadex beds were prewashed with KR-H for 30 min at 37 °C, after which time a constant spontaneous outflow of radioactivity was attained. Then 0.7 ml fractions (one per 120 s) were collected directly into scintillation vials. With the 4th to the 5th fraction 700  $\mu l$  KR-H containing 25 mM  $K^+$  (its sodium content had been reduced by the same amount) reached the column. A total of 7 fractions was taken, mixed with 5 ml Instagel II each, and the radioactivity was measured. Except in the choline experiments the Sephadex bed containing the particles was removed from the column at the end of the experiment and its radioactivity determined. The evoked release was calculated as a fraction of total radioactivity present at the start of depolarization (for choline see Bigalke et al. 1981). Data from unpoisoned controls for glycine, GABA and noradrenaline are given in Table 1.

Within a single series the variances in the evoked release remain acceptably low i.e. in the range of  $\pm 10\%$ . The losses of radioactivity between filling the columns (Table 1, II) and start of the collection period (Table 1, III) depend on the speed of the experimenter and are difficult to standardize. Therefore we preferred to express the evoked release not in absolute values but as percentage of radioactivity present at the start of the collection period within the individual sample. Comparisons between poisoned and non-poisoned samples (both in triplicates) were always made within a single series consisting of twelve samples.

We also measured by a filtration technique the transmitter content at the start and the end of the poisoning period in order to exclude any toxin effect on the transmitter content of preloaded particles before superfusion. Spinal cord particles were preloaded for 60 min with labelled glycine (1.5  $\mu M$ ), GABA (1  $\mu M$ ) or noradrenaline (0.2  $\mu M$ ), and striatal particles with noradrenaline (0.2  $\mu M$ ). Thereafter they were washed, incubated for 2 h with tetanus toxin (1 and 0.1  $\mu g/ml$ ), botulinum A toxin (10  $\mu g/ml$ ) or KR-H, and filtered as described in the uptake experiments. The radioactivity decreased during the 2 h incubation by 20% (noradrenaline) and 50% (glycine), however independently of the presence of the toxins.

In short-time experiments similar to those described by Kryzhanovsky et al. (1980), tetanus toxin also did not enhance noradrenaline release.

To identify the radioactive material, particles were preincubated first with the labelled transmitters as given in Table 1, then with KR-H-alb for 2 h, and washed twice with KR-H. The final sediments were resuspended in 200  $\mu l$  95% ethanol, homogenized by sonication (Branson Sonifier) in an Eppendorf vessel and centrifuged. Samples of the supernatants were counted for radioactivity or subjected, together with standards, to thin-layer chromatography on cellulose plates (Merck, Darmstadt, FRG). The plates were run with *n*-butanol (12)-acetic acid (3)- $H_2O$  (5). The spots were rendered visible with ninhydrin and the lanes scanned using a Berthold radioscaner. Radioactivity always coincided with the re-

**Table 1.** Calculation of spontaneous and potassium evoked release from spinal cord (glycine), forebrain (GABA) and striatal (noradrenaline) particles. Eleven or twelve samples of particulate preparations from spinal cord, forebrain and corpus striatum were incubated with the labelled transmitter (glycine: 78,240 dpm/sample, GABA: 689,460 dpm/sample, noradrenaline: 601,380 dpm/sample), processed as usual and superfused (see Bigalke et al. 1981)

Transmitter and particles studied (I)	Glycine from spinal cord (11)	GABA from forebrain (12)	Noradrenaline from C. striatum (12)
Radioactivity before superfusion (II)	15,800 ± 900	192,000 ± 14,700	209,300 ± 6,300
Radioactivity at the start of the collection period (III)	7,900 ± 400	142,000 ± 14,400	147,700 ± 9,400
Total release during the collection period (IV)	3,000 ± 100	39,100 ± 3,200	57,700 ± 3,300
Evoked release (V)	800 ± 100	18,000 ± 2,200	42,100 ± 2,700
Evoked release in percent of radioactivity at the start of collection calculated for the individual samples (VI)	10.3 ± 0.9	12.7 ± 0.8	28.5 ± 2.1

All values are given as dpm ± SD. During the washout period the particles lost radioactivity as shown by the differences between lines II and III. The radioactivity at the start of the collection (line III) is the sum of the total release during collection plus the radioactivity remaining in the columns at the end of the superfusion (see Methods). Total release during collection (line IV) consists of basal and evoked release. The evoked release (line V) was calculated by subtracting the basal release (for calculation see Bigalke et al. 1981) from the total release. Line VI gives again the evoked release, however calculated for the individual samples as fraction (%) of radioactivity at the start of collection ( $\bar{x} \pm \text{SD}$ ). Each vertical column represents one assay with the number of samples given in line I

spective unlabelled transmitters. Since the radioactive material in the eluants was not identified, the release can be given as radioactivity only, and not as units of weight.

## Results

### 1. Uptake

All uptake studies have been performed with crude synaptosomal fractions, in order to allow a direct comparison with our previous studies on the cholinergic system (Habermann et al. 1981).

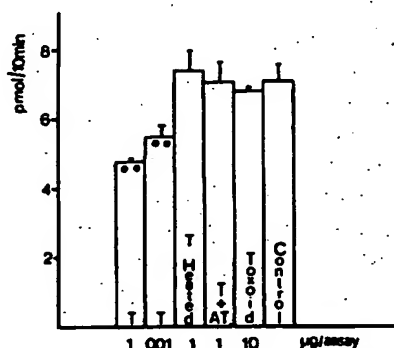
Glycine uptake is partially inhibited in a dose-dependent manner by preincubation with tetanus toxin (Fig. 1) but hardly and unreproducibly by botulinum A toxin. Like choline uptake into rat cerebral cortex synaptosomes (Habermann et al. 1981), inhibition of glycine uptake by tetanus toxin becomes statistically significant after a lag period longer than 1 h (Fig. 2). Uptake into non-poisoned synaptosomes also declines in a time-dependent manner, however to a smaller degree. Decrease in accumulation is not due to enhanced loss of glycine once taken up (see Methods).

Inhibition of glycine uptake is due to tetanus toxin and not to a contaminant because a) toxoid is ineffective, b) previous heating destroys the inhibitory potency, and c) antibodies neutralize it (Fig. 1). The very weak and irregular effect of botulinum A toxin was abolished by heating and could be reproduced by its neurotoxin (not shown).

A weak inhibition of GABA uptake into forebrain cortex synaptosomes became statistically significant over a wide range of tetanus toxin concentrations.

The effect was specific because it was absent with antibody- or heat-treated toxin (not shown). However, toxoid also diminished the GABA uptake, provided it was applied in concentrations far above those chosen for toxin (Fig. 3).

No effect of botulinum or tetanus toxin (up to 1 µg/assay) was observed on the noradrenaline uptake into striatal synaptosomes. Dopamine uptake into striatal synaptosomes and 5-hydroxytryptamine uptake into cortical synaptosomes was only negligibly influenced by either toxin (not shown).



**Fig. 1.** Glycine uptake into rat spinal cord synaptosomes: Identity tests for tetanus toxin. The first incubation lasted 2 h at 37 °C, the second 10 min at 30 °C. The final concentration of glycine added was  $2.5 \times 10^{-6}$  M. The ordinate represents the uptake per sample (420 µg protein). Toxin (1 µg and 0.01 µg, T) decreases the uptake as compared with the untreated sample (Control). Toxin (1 µg) heated to 95 °C for 10 min or treated with 10 U dialyzed antitoxin (AT), or dialyzed tetanus toxoid (10 µg) are ineffective. Dialyzed antitoxin did not alter the uptake when applied alone.  $\bar{x} \pm \text{SD}$ ;  $n = 3$ . \*\*  $P = 1-2\%$ .

### 2. Release

**Characterization of the Release Process.** Independently of the transmitter and the part of the central nervous system studied, the release followed a common pattern. After the initial washout, leakage of radioactivity (expressed in percent of total radioactivity present) became nearly constant over the resting period. Depolarization with 25 mM  $\text{K}^+$  for 2 min increased the radioactivity released in a calcium dependent manner, as shown for GABA on forebrain, for noradrenaline on striatum, and for glycine in the spinal cord (Fig. 4).

The evoked release depended on the potassium concentration applied (Fig. 5). We selected a suboptimal concentration of potassium because the effects of tetanus and botulinum A toxin on acetylcholine release had been partially overcome by raising the potassium concentration (Bigalke et al. 1981).

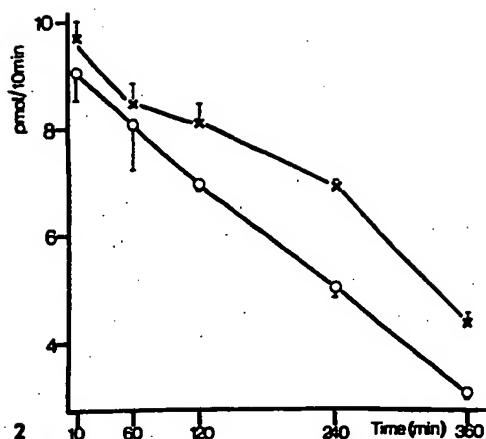


Fig. 2. Glycine uptake into rat spinal cord synaptosomes: Lag period of the effect of tetanus toxin. The time of the first incubation, without (x) or with (O) 100 ng tetanus toxin per assay, was varied between 10 min and 360 min at 37°C, whereas the second incubation was kept constant (see Fig. 1). Each symbol represents the arithmetic mean of 3 determinations  $\pm$  SD. Ordinate refers to uptake into 280  $\mu$ g synaptosomal protein.  $\bar{x} \pm$  SD;  $n = 3$

Fig. 3a and b. GABA uptake into rat forebrain synaptosomes. The first incubation lasted 2 h at 37°C, the second 3 min at 37°C. GABA concentration was 0.7  $\mu$ M, and the protein content was 600  $\mu$ g/sample. The numbers on the bars represent the amounts of tetanus toxin (T), tetanus toxoid, or botulinum A toxin (B) applied. C = toxin-free control. All values are the means of triplicates  $\pm$  SD. a Effects of tetanus toxin. \*\*  $P = 0.5\%$ . b Minimal effects of toxoid, no effects of botulinum toxin. \*  $P = 2-5\%$ .

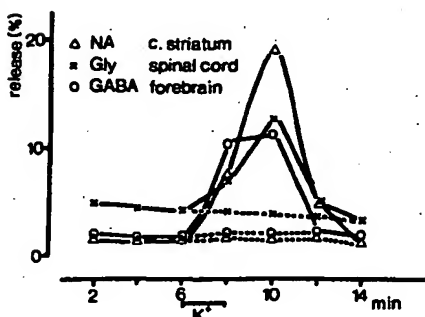
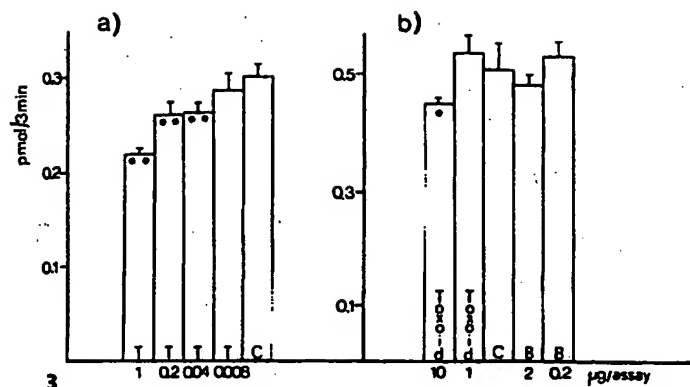


Fig. 4. Calcium-dependence of the evoked (25 mM  $K^+$ ) release of radioactivity. After a 30 min superfusion period the release from forebrain (GABA, O), corpus striatum (noradrenaline,  $\Delta$ ) and spinal cord (glycine, x) had reached a steady base line. The particles released additional radioactivity due to 25 mM potassium in the presence of  $Ca^{2+}$  but not in its absence (dotted lines). Release is expressed as percentage of total radioactivity present before collecting

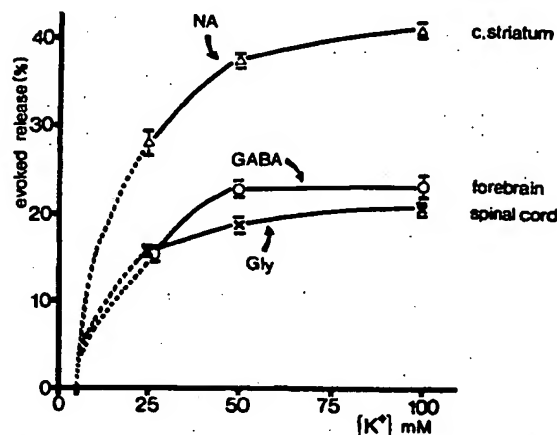


Fig. 5. Dependence on potassium concentration of the release. The particles were preloaded with GABA (forebrain, O), glycine (spinal cord, x) and noradrenaline (striatum,  $\Delta$ ), incubated for further 120 min in KR-H and then superfused as described under Methods. They were depolarized for 120 s with different potassium concentrations. The sodium content had been decreased by the same amount. Evoked release is given as percentage of the total radioactivity present in the particles before collecting.  $\bar{x} \pm$  SD,  $n = 3$

The percentage of radioactivity released depended also on the part of the central nervous system used. Arguments for the specificity of the release process may be drawn from the observations that potassium fails to release radioactivity from the non cholinergic cerebellum preloaded with [ $^3$ H]-choline; that the evoked release of the non-transmitter serine from spinal cord was minimal and that the evoked release was  $Ca^{2+}$  dependent (Fig. 4).

**Inhibition by Tetanus and Botulinum Toxin of the Release Process.** Tetanus toxin inhibits the release of radioactivity from spinal cord particles preloaded with labelled glycine, GABA, noradrenaline and acetylcholine (Fig. 6a). The degree of inhibition depends on the toxin concentration and may exceed 95% with glycine, 70% with acetylcholine and GABA, and 60% with noradrenaline. With respect to in-

hibitory transmitter amino acids tetanus toxin inhibits the release in amounts of a few mouse  $LD_{50}$  per ml.

In forebrain cortex particles, glycine and GABA are again most sensitive to tetanus toxin, whereas about 100 times higher toxin concentrations are required for inhibition of acetylcholine release (Fig. 6b). Release from striatal particles preloaded with noradrenaline was also less sensitive than glycine and GABA release from forebrain (Fig. 6b).

As with the inhibition of acetylcholine release from rat forebrain slices (Bigalke et al. 1981), botulinum toxin is considerably less potent than tetanus toxin with any transmitter studied in spinal cord, striatal and forebrain particles (Fig. 7). Release from striatal particles preloaded with noradrenaline is sensitive to botulinum A toxin in the same dose range as is acetylcholine release from forebrain. In any case, concentrations of 1–20  $\mu$ g/ml of botulinum A toxin are

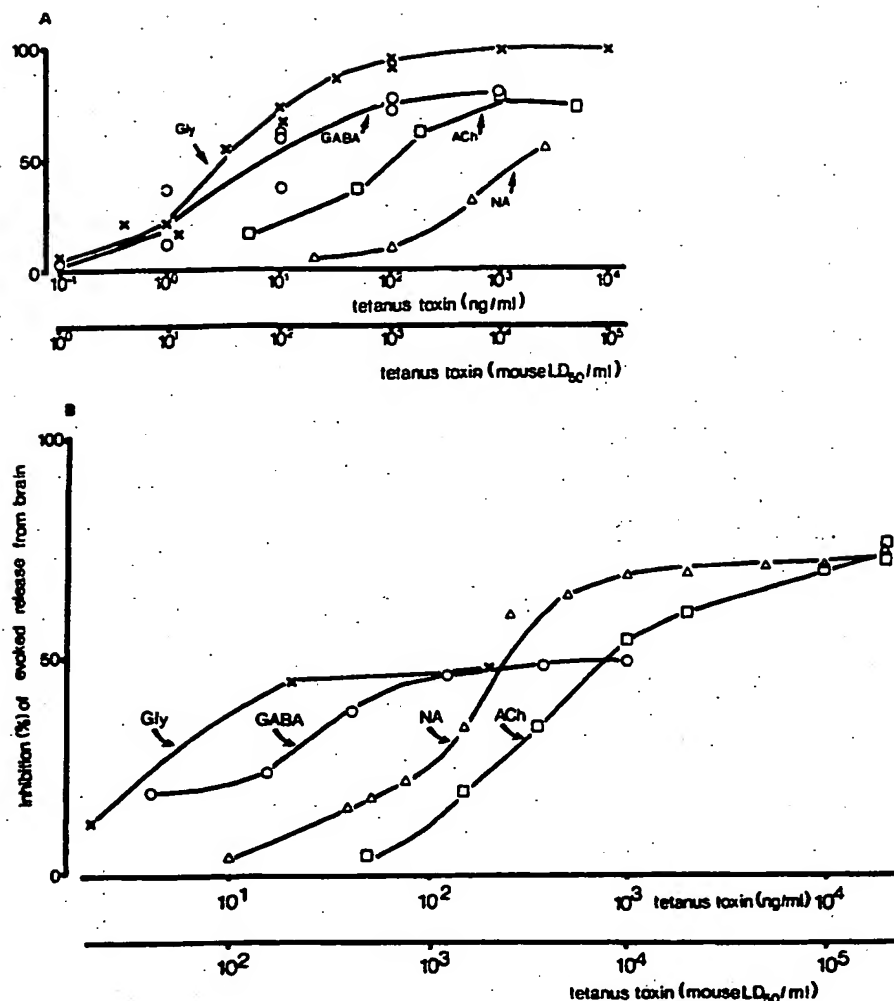


Fig. 6a and b  
Inhibition by tetanus toxin of the evoked release. Ordinate: inhibition (%) of evoked release. Abscissa: concentration of tetanus toxin in ng/ml (upper scale) and mouse LD<sub>50</sub>/ml (lower scale). Inhibition was calculated using the quotient of the means of three poisoned and three unpoisoned samples. The same formula was used as previously (Bigalke et al. 1981):

$$100 - \frac{\text{evoked release from toxin-treated particles}}{\text{evoked release from reference particles}} \times 100$$
 Glycine (x—x), GABA (O—O), acetylcholine (□—□), noradrenaline (Δ—Δ). (a) Spinal cord particles. (b) Forebrain (glycine, GABA and acetylcholine) and striatal (noradrenaline) particles

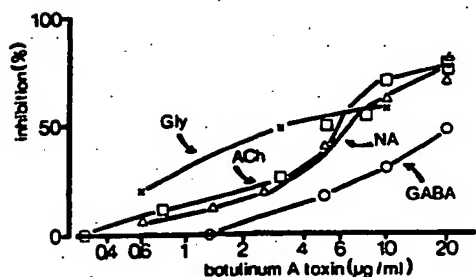


Fig. 7. Inhibition by botulinum A toxin of the evoked release from particles of the central nervous system. For details see legend to Fig. 6. Forebrain particles were preloaded with choline or GABA, spinal cord particles with glycine, and striatal particles with noradrenaline. The abscissa gives the toxin concentration (μg/ml). 1 μg/ml is equivalent to about 10<sup>4</sup> mouse LD<sub>50</sub>/ml

required, and the same dose range is needed for inhibition of glycine release from spinal cord. In contrast tetanus toxin (see Fig. 6) dose-dependently depresses the release from forebrain and spinal cord particles preloaded with glycine and GABA already when 1–100 ng/ml toxin are applied.

**Inhibition of Release is due to Botulinum and Tetanus Neurotoxins.** Already the considerable potency of the tetanus toxin sample argues against the involvement of a con-

tamination. The linkage between toxin and inhibitory power was substantiated by three additional controls with glycine, GABA and noradrenaline on spinal cord, forebrain and corpus striatum respectively. Heated toxin, toxoid, and toxin pretreated with antitoxin were inactive as compared with an untreated toxin sample (Table 2). Tetanus antitoxin itself had no effect on the release processes studied (not shown).

The causative role of *botulinum A* toxin was established in a similar manner. Previously we (Bigalke et al. 1981) had shown that the inhibitory effect of the toxin on acetylcholine release from forebrain slices was abolished by its mixing with antitoxin or by previous heat treatment. Now the inhibition of release from Corpus striatum particles preloaded with noradrenaline was also found to be abolished by antitoxin. Botulinum antitoxin itself slightly diminished the release after preloading with GABA (Table 3) and with glycine (not shown) which complicates the interpretation of the neutralization experiments.

The low efficacy of botulinum A toxin leads to the question whether its effects are due to its neurotoxin or to its haemagglutinin content. Haemagglutinin was not available. The stock of neurotoxin had lost some toxicity during storage, its LD<sub>50</sub> had increased from 4 ng/kg (see Habermann et al. 1981) to 7.5 ng/kg (see Methods) which is in the same range as the LD<sub>50</sub> of "crystalline" botulinum A toxin. Accordingly, the neurotoxin preparation was about equipotent

Table 2. Identity tests for the tetanus toxin effect on the release of radioactivity from particles preloaded with noradrenaline (corpus striatum), GABA (forebrain) and glycine (spinal cord). Preloaded particles were incubated for 120 min with KR-H, tetanus toxin, or inactivated tetanus toxin. The evoked release after preloading with noradrenaline, GABA and glycine was depressed by the high concentration of tetanus toxin used. Inactivated tetanus toxin was without effect. Release is expressed as percentage of total radioactivity present at the start of depolarization ( $\bar{x} \pm SD$ ,  $n = 3$ ). Each column (a, b, c) represents a single experimental series. — = Not tested

Transmitter	Treatment	Dosage of		Evoked release (%)		
		Toxin ( $\mu\text{g/ml}$ )	Antitoxin (U/ml)	a	b	c
Noradrenaline	No toxin			$32 \pm 3$	$31 \pm 2$	—
	Toxin	5		$7 \pm 2$	$10 \pm 1$	—
	Heated toxin	10		$35 \pm 1$	—	—
	Toxin and antitoxin	5	50	—	$32 \pm 4$	—
	Toxoid	40		$30 \pm 2$	—	—
GABA	No toxin			$20 \pm 2$	$16 \pm 1$	—
	Toxin	10		$9 \pm 1$	—	—
	Toxin	0.1		—	$9 \pm 1$	—
	Heated toxin	10		$19 \pm 2$	—	—
	Toxin and antitoxin	0.1	100	—	$16 \pm 1$	—
	Toxoid	100		$20 \pm 0$	—	—
Glycine	No toxin			$12 \pm 1$	$14 \pm 2$	$11 \pm 1$
	Toxin	0.05		$3 \pm 1$	$2 \pm 1$	$2 \pm 0$
	Heated toxin	1		—	$14 \pm 2$	—
	Antitoxin		100	$11 \pm 1$	—	—
	Toxin and antitoxin	1	100	$10 \pm 2$	—	—
	Toxoid	10		—	—	$10 \pm 0$

Table 3. Inhibition of release by botulinum A neurotoxin, and abolishment of the botulinum A toxin effects by heating and antitoxin. Preloaded particles were incubated for 120 min with KR-H, botulinum toxin, neurotoxin or inactivated toxin. The evoked release was partially prevented with botulinum toxin and neurotoxin. Heated or antitoxin-treated toxins were without effect. Release is expressed as percentage of total radioactivity present at the start of depolarization ( $\bar{x} \pm SD$ ,  $n = 3$ ). SD of 0 indicates  $< 0.5$ . Columns a and b indicate two different experiments. — = Not tested. Antitoxin had a slight effect of its own on GABA release

Transmitter	Source of particles	Treatment	Doses of		Evoked release	
			Toxin ( $\mu\text{g/ml}$ )	Antitoxin (U/ml)	a	b
Glycine	Spinal cord	No toxin	—	—	$17 \pm 1$	—
		Neurotoxin	3	—	$10 \pm 0$	—
		Heated neurotoxin	3	—	$17 \pm 1$	—
GABA	Forebrain	No toxin	—	—	$16 \pm 2$	—
		Antitoxin	—	50	$13 \pm 0$	—
		Toxin	20	—	$9 \pm 1$	—
		Toxin with antitoxin	20	50	$13 \pm 3$	—
Noradrenaline	Corpus striatum	No toxin	—	—	$36 \pm 2$	$20 \pm 1$
		Botulinum A toxin	10	—	$9 \pm 1$	—
		Toxin with antitoxin	10	25	$36 \pm 2$	—
		Neurotoxin	6	—	—	$11 \pm 1$
		Heated neurotoxin	6	—	—	$20 \pm 0$

fective with botulinum A toxin with respect to inhibition of noradrenaline release (Table 3). Heat treated neurotoxin no longer inhibited the release as studied with glycine or noradrenaline.

## Discussion

The observations presented confirm and extend those reported previously (Habermann et al. 1981; Bigalke et al. 1981) for the cholinergic system. We shall now try a synopsis on the basis of the following three assertions.

### 1. Central Synapses are Better Targets for Tetanus Toxin. Whereas Peripheral Cholinergic Synapses are more Sensitive to Botulinum A Toxin

We may arrange various pharmacological systems as to their relative sensitivities towards botulinum A and tetanus toxin. The neuromuscular junction of the mouse diaphragm is extremely sensitive to botulinum toxin, and about 1,000 times less to tetanus toxin (Habermann et al. 1980b). Due to decreased sensitivity to botulinum toxin, the quotient is diminished to about 5 in the ileal muscle strip of the guinea-



pig (Bigalke and Habermann 1980). On cholinergic nerve endings from the central nervous system, the quotient is decreased to 0.1, due to the further decreased efficacy of botulinum toxin (Bigalke et al. 1981). The quotient becomes still smaller with regard to other neurotransmitters (this communication). For instance it is about 0.001 for release from brain and spinal cord particles preloaded with glycine or GABA, due to an increase in tetanus toxin sensitivity. Thus the discrimination index between the two toxins is between  $10^3$  and  $10^6$  when compared on the mouse diaphragm and on the amino acid release. So far unexplained is the difference between slices and particles on the one hand, where tetanus toxin is more potent in inhibiting acetylcholine release, and primary cell cultures on the other where botulinum toxin was more potent (Bigalke et al. 1978). However, in the former systems, we have measured the radioactivity released, whereas in cell cultures the radioactivity retained had to be determined for technical reasons.

## 2. Tetanus and Botulinum A Toxin are not Specific as to the Central Transmitter Systems Studied

In peripheral organs, tetanus and botulinum A toxin appear to be directed specifically to cholinergic synapses. We (Habermann et al. 1980a) were not able to confirm earlier claims concerning the efficacy of botulinum A toxin on the peripheral adrenergic system. In the central nervous system, transmitter specificity of either toxin is lost. Botulinum A toxin was about equipotent as to inhibition of release as studied with acetylcholine, noradrenaline and glycine, and it also inhibited the release from particles preloaded with GABA when given in higher concentrations. Conversely tetanus toxin depressed the release with all transmitters studied, not only with acetylcholine (Bigalke et al. 1981), but also with glycine, GABA, and noradrenaline. The same applied for the uptake studies where tetanus toxin diminished not only the accumulation of choline (Habermann et al. 1981) but also of glycine and GABA. Restricted specificity is evident also from the findings of Osborne and Bradford (1973) who showed the release of more than one amino acid to be depressed by tetanus toxin, and from those of Collingridge et al. (1980) who reported the release of two different transmitters – GABA and dopamine – to be impaired by in vivo application of tetanus toxin into the nigrostriatal system. The last-mentioned authors did not find any effect of tetanus toxin on transmitter uptake, or on the release of acetylcholine and 5-hydroxytryptamine. The ways of poisoning are probably too different to allow direct comparisons with our data. Whereas Collingridge et al. (1980) poisoned in vivo and took material from the area infiltrated, we exposed particles to defined toxin concentrations in vitro. In that way, we might have exposed the synapses to the toxins more homogeneously.

Uptake and release may be inhibited either directly or indirectly. With decreased uptake, less transmitter might be available for release. We escaped this problem by preloading the particles with radioactivity. During subsequent poisoning, some radioactivity was lost, however not more than from the toxin-free sample. Therefore decreased release is not due to decreased uptake, a conclusion also reached by Collingridge et al. (1980). Moreover, the uptake of some transmitters (for instance of noradrenaline) was not depressed measurably by the toxins, although their release was inhibited.

Even when the uptake was toxin-sensitive, release was so much more.

The present contribution does not give any hint how the toxins act at the molecular level. The multiplicity of the effects suggests that the target of both toxins must be very common to many, perhaps all central neurones of mammals. An explanation is to be sought why the suppression of uptake and release is only partial.

## 3. Inhibition of Central Transmitter Systems is Relevant in Tetanus but not in Botulism

It is generally accepted that neuromuscular and intestinal cholinergic paralysis are prominent in botulism. In contrast, botulinum toxin effects on the central nervous system, albeit present (see Wiegand and Wellhöner 1977) are negligible for two reasons. The neuronal ascent of the toxin is modest as compared with tetanus toxin (Habermann 1974; Wiegand et al. 1976), and its potency is much weaker, at least on particles. Accordingly intrastriatal injections of botulinum A toxin in rats were tolerated without local symptoms (unpublished experiment).

The situation is different for tetanus toxin. For many years there was a gap between the alleged specificity of tetanus toxin on certain inhibitory synapses, and the allround efficacy of tetanus toxin in the central nervous system. Kryzhanovsky and Aliev (1976) applied tetanus toxin to many areas of the nervous system in order to raise local foci which they called "universal dispatch stations". Our findings underline the particular sensitivity of inhibitory, for instance glycinergic and GABA-ergic systems. However, the other, less sensitive adrenergic and cholinergic systems should not be neglected, and also not the sensitivity of the dopaminergic system described by Collingridge et al. (1980). Wellhöner (1981) has listed many spinal and supraspinal functions as targets of tetanus toxin. Besides of the relative sensitivity, it is a matter of application and of the pharmacokinetic circumstances, for instance the migration of tetanus toxin into or within the nervous system (see Habermann 1978) which transmitter system will be affected in the first instance.

Taken all our data together (Habermann et al. 1980a, b; Bigalke and Habermann 1980; Bigalke et al. 1981; Habermann et al. 1981; this communication), considerable quantitative differences exist between the efficacy of tetanus and botulinum toxin on various transmitter systems in vitro and in vivo. However, there were no differences in qualitative terms.

**Acknowledgements.** We are indebted to Ms. Eva Bolz, Monika Laux and Mr. Helmut Müller for their skillful technical assistance. Work was supported by the Deutsche Forschungsgemeinschaft (SFB 47) and by the Bundesministerium des Innern.

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Received January 22/Accepted March 23, 1981

*Note Added in Proof.* 1. After acceptance of our manuscript, an abstract (Collingridge GL, Davies J (1980) *J Physiol* 308:72P–73P) came to our knowledge. It reported a decrease of potassium evoked release of [ $^3$ H]-GABA from slices preloaded and then poisoned in vitro, which is completely in accord with our data. The authors saw no effect on [ $^3$ H]-GABA uptake into slices whereas we found a minor diminution of uptake into particles (see Fig. 2). 2. In still unpublished experiments from this laboratory F. Dreyer and A. Schmitt found the neuromuscular transmission to be inhibited in a different manner by tetanus or botulinum toxin, although the macroscopical features of in vitro paralysis are very similar.